Committee:
Dr. Gardner
Dr. Liebow
Dr. Sommers

CHRONIC PULMONARY DISEASES

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N.Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

 Date:

1. Principal Investigator (give title and degrees):

Patrick E. Lentz, Ph.D.

2. Institution & address:

Tulane University School of Medicine
1430 Tulane Avenue
New Orleans, Louisiana 70112

3. Department(s) where research will be done or collaboration provided:

Department of Physiology

4. Short title of study:

Cigarette Smoke and Transport of Substrates by Alveolar Macrophages

5. Proposed starting date: January, 1974

6. Estimated time to complete: 36 months

7. Brief description of specific research aims:

Recent studies in this laboratory have demonstrated that aqueous extracts of cigarette smoke inhibit the ability of alveolar macrophages to actively transport the non-metabolizeable amino acid, α amino isobutyric ^{14}C- (AIB). The aim of this research program is to examine the mechanisms whereby certain components of cigarette smoke interfere with the transport of AIB across the membrane of alveolar macrophages.

The specific objectives of this investigation are:

1) to establish the dose response and time course relationships for the effects of aqueous extracts of cigarette smoke (AECS), nicotine, and cadmium on the active accumulation of a non-metabolizeable amino acid, AIB, by alveolar macrophages
2) to determine the effects of AECS, nicotine, and cadmium on the kinetic parameters of active accumulation of AIB
3) to study the relationship between the effects of AECS, nicotine, and cadmium on AIB transport and the concentration of ATP in alveolar macrophages
4) to study the relationship between the effects of AECS, nicotine or cadmium on AIB transport, and alteration of the nature or active site of the carrier.

8. Brief statement of working hypothesis:

Alveolar macrophages are a vital component of the pulmonary host defense systems. These cells are responsible for phagocytosis, killing and degradation of bacteria which penetrate to the alveolar surface. Since the functional integrity of the alveolar macrophage depends, to a certain extent, on the availability and membrane transport of essential substrates, our observations of a depression in active transport after exposure to extracts of cigarette smoke may have important clinical significance in pulmonary disease states associated with inhalation of cigarette smoke.

I have formed the working hypothesis that certain components of cigarette smoke impair the ability of alveolar macrophages to actively transport an amino acid either by decreasing the quantity of binding sites or carrier molecules in the cell membrane or by reducing the energy supply available for transport.

9. Details of experimental design and procedures (append extra pages as necessary)

See attached pages.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Adequate laboratory and office space are provided by the Department of Physiology. Facilities for purchase and maintenance of rabbits are available in the Department of Vivarial Science, Tulane University School of Medicine.

Major items of equipment available in this Department include: one Filtrona Smoking Machine, one PR-6 Refrigerated Centrifuge, one Nuclear Chicago Liquid Scintillation Counter, one Beckman DBG Spectrophotometer, microscopes, vacuum oven.

11. Additional facilities required:

An additional Dubnoff shaking water bath is requested.

12. Biographical sketches of investigator(s) and other professional personnel (append):

CV append.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Append list and reprints.

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PATRICK EDMUND LENTZ, Ph.D.

BIRTHDATE: REDACTED
PLACE:
Married

EDUCATION:

Institution	Date	Degree
Loras College Dubuque, Iowa		Bachelor of Arts, Biology
Marquette University Milwaukee, Wisconsin		Master of Science, Medical Physiology
Marquette University Milwaukee, Wisconsin		Completion Academic Requirements for Doctor of Philosophy
University of Wisconsin Madison, Wisconsin		Special Student (Doctoral research with Dr. J. L. Van Lancker
Brown University Providence, R.I.		Special Student
Marquette University Milwaukee, Wisconsin		Doctor of Philosophy Degree in Medical Physiology

POSITIONS:

Brown University Providence, R.I.	1967-1968	Research Associate, Division of Biological and Medical Science
Tulane University School of Medicine, New Orleans	1969-	Assistant Professor, Department of Physiology

FELLOWSHIPS:

1. Predoctoral Trainee, United States Public Health Service, 1962-1966.
2. Predoctoral Fellowship, United States Public Health Service, 1966-1967.

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PROFESSIONAL SOCIETIES:

REDACTED

RESEARCH EXPERIENCE:

1962-1964	Research on the role of the functional status of the reticuloendothelial system in the pathogenesis of hemorrhagic shock.
1964-1967	Research on the site of synthesis of beta glucuronidase in regenerating rat liver.
	(a) studies on the site of synthesis and transfer of beta glucuronidase from the microsomal to the small mitochondrial or lysosomal fraction.
	(b) comparison of the catalytic properties of beta glucuronidase purified from microsomal and small mitochondrial fractions.
1967-1968	1. Research on the localization, mode of attachment and release of beta glucuronidase from microsomal sub-fractions.
	2. Research on the site of synthesis of glutamic dehydrogenase.
1969-1970	1. Biochemical characterization of parenchymal and Kupffer cells isolated from rat liver.
1971-present	1. Synthesis of RNA, DNA and protein by parenchymal and Kupffer cells isolated from rat liver.
	2. Effect of actinomycin D, chloromphenicol and puromycin on nucleic acid and protein synthesis in parenchymal and Kupffer cells isolated from rat liver.
	3. Specificity and immunogenicity of RNA synthesized in macrophages after exposure to antigens, such as sheep erythrocytes, E. coli and BP-4 tumor cells.

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4. Metabolic, phagocytic and bactericidal activities of alveolar macrophages exposed to air pollutants such as cigarette smoke.

RESEARCH INTEREST:

1. Physiological role of parenchymal and Kupffer cells in liver metabolism.

2. Effects of nutritional status, and drugs on metabolism of liver Kupffer and parenchymal cells.

3 Effects of air contaminants (cigarette smoke, oxides of nitrogen and sulfur) on metabolic and bactericidal activities of pulmonary alveolar macrophages.

4. Molecular mechanisms of the immune response. Role of macrophage RNA in the initiation of an immune response to specific antigens.

PUBLICATIONS:

Lentz, Patrick E., James M. Lipo and William J. Stekiel. Effect of hemorrhagic shock on rat plasma lysosomal hydrolase activity. Fed. Proc. 24, 1965 (Abstract).

Lentz, Patrick E. and James J. Smith. The effect of reticuloendothelial stimulation on plasma hydrolase activity in hemorrhagic shock. Proc. Soc. Exp. Biol. Med. 124:1243-1248, 1967.

Lentz, Patrick E. Studies on the Site of Synthesis of Beta glucuronidase. Ph.D. Thesis, Marquette University, 1967.

Lentz, Patrick E. and J. L. Van Lancker. The transfer of beta glucuronidase from the microsomal to the small mitochondrial fraction in hypoxic livers. Am. J. Pathol. 1968 (Abstract).

Van Lancker, J. L. and Patrick E. Lentz. Molecular mechanisms of liver regeneration. VI. The site of synthesis of beta glucuronidase. J. Histochemistry and Cytochemistry. 18:529-541, 1970.

Van Lancker. J. L., V. Melnick and P. E. Lentz. Site of synthesis of glutamic dehydrogenase in regenerating rat liver. (manuscript in preparation).

Lentz, Patrick E. and N. R. Di Luzio. Biochemical characteristics of parenchymal and Kupffer cells isolated from rat liver. Exp. Cell Res. 67:17-26, 1971.

Lentz, P. E. and N. R. Di Luzio. Isolation of immunogenic RNA from liver, splenic and peritoneal macrophages (accepted RES - J. Reticuloendothelial Soc., 1973).

Lentz, P. E. and N. R. Di Luzio. Effect of AET (B-amino ethyl isothiouranium) and isologous bone marrow on reticuloendothelial function after cobalt-60 irradiation (submitted for publication Radiation Research).

Lentz, P. E. and N. R. Di Luzio. Biochemical composition of Kupffer and parenchymal cells isolated from normal, RE-stimulated or depressed rats. RES, J. Reticuloendothelial Society 9:609, 1971 (Abstract).

Lentz, P. E. Isolation of immunogenic RNA from rat macrophages. Abstract of paper presented at Fall Meeting American Physiology Society, August, 1971.

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Lentz, P. E. Bactericidal and metabolic activities of alveolar macrophages exposed to tobacco smoke. Abstract of paper presented at Eighth National Meeting of the Reticuloendothelial Society, Detroit, Michigan, November 30, 1971.

Lentz, P. E. and N. R. Di Luzio. Comparative influence of aqueous extracts of cigarette smoke on phagocytic, bactericidal and metabolic activities of macrophages. Abstract of paper presented at symposium sponsored by American Medical Association Education and Research Foundation, May, 1972.

Pisano, J. C., J. T. Patterson, R. Trejo, E. Hoffmann, P. E. Lentz and N. R. Di Luzio. Hepatotoxic effects of horse anti-mouse lymphocyte serum. Exp. Molecular Pathology 16:302, 1972.

Lentz, P. E. and N. R. Di Luzio. Phagocytic and Bactericidal Activities of rat alveolar macrophages exposed to aqueous extracts of cigarette smoke in vitro. (submitted Am. Rev. Resp. Disease).

Lentz, P. E. Effects of actinomycin D, puromycin and chloramphenicol on nucleic acid and protein synthesis by Kupffer and parenchymal cells isolated from rat liver. Rush Presbyterian St. Luke's Medical Bulletin 12:211-221, 1973.

Lentz, P. E. and N. R. Di Luzio. Isolation of adult rat liver macrophages (von Kupffer cells). in Biomembranes (Cells, Organelles and Membraneous Components). A volume of Methods in Enzymology. ed. S. P. Colowick and N. O. Kaplan. Academic Press, Inc. New York, NY, Accepted for publication.

Lentz, P. E. and N. R. Di Luzio. Functional alterations in alveolar macrophages exposed to cigarette smoke in vitro and in vivo. Abstract of paper to be presented at 9th National Meeting of the Reticuloendothelial Society. December, 1972.

Lentz, P. E. and N. R. Di Luzio. Transport of alpha aminoisobutyric acid by alveolar macrophages incubated with cigarette smoke and nicotine. (Submitted Arch. Environ. Health, 1973).

Lentz, P. E. and N. R. Di Luzio. Peroxidation of lipids in alveolar macrophages and pulmonary protective factor by aqueous extracts of cigarette smoke (submitted Arch. Environ. Health, 1973).

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Patrick E. Lentz, Ph.D. Principal Investigator	22	REDACTED
Fringe Benefits		
Technical		
Research Technician, Grade III	100	REDACTED
Fringe Benefits		
		REDACTED
Sub-Total for A		

B. Consumable supplies (by major categories)

Animals 160 rabbits @ $10 ea.	1,600.00
Isotopes α-aminoisobutyric acid -1-^{14}C	600.00
inulin carboxy ^{14}C	
Scintillation counting supplies	800.00
Laboratory chemicals and glassware	600.00
Cigarettes	100.00
Sub-Total for B	3,700.00

C. Other expenses (itemize)

Travel one National Meeting	300.00
Sub-Total for C	300.00
Running Total of A + B + C	17,588.00

D. Permanent equipment (itemize)

1 Dubnoff Shaking Water Bath	400.00
Sub-Total for D	400.00
E	2,638.00
Total request	20,626.00

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	REDACTED	3,700.00	300.00	-0-	2,753.00	$21,104.00
Year 3		3,700.00	300.00	-0-	2,886.00	$22,126.00

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Transport of Glucose By Alveolar Macrophages	National Heart and Lung Institute	30,000.	2 years July 1974-76

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Patrick E. Lentz, Ph.D.

Signature Patrick E Lentz R Date 11/7/73

Telephone ___ ___ ___ ___
Area Code Number Extension

Checks payable to

Tulane University

Mailing address for checks

Jesse B. Morgan
Business Manager and Comptroller
Tulane University
New Orleans, La.

Responsible officer of institution

Typed Name J. T. Hamlin, III, M.D.

Title Vice-Dean

Signature [signature] Date 11/7/73

Telephone ___ ___ ___ ___
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I. Amino Acid Transport in Mammalian Cells

The cell membrane has the capacity to transport solutes into and out of the cell. These transport processes are responsible for maintaining the constancy of the internal environment of the cell and providing the substrates required for cellular metabolism, and synthetic activities. Within the last decade the molecular mechanisms underlying membrane transport have begun to be unraveled, and certain characteristics of the transport process are now well established. Kinetic studies with bacterial and mammalian cells have provided convincing evidence for mobile membrane carrier systems with variable degrees of specificity for a variety of solutes. Unfortunately, the chemical nature of the carrier has not been established, and until such data are available, there will be a considerable gap in our understanding of the precise mechanism of operation of the transport system.

This review of the literature will be restricted to: 1) the active transport of amino acids in animal cells; the many interesting features of iorganic ion, water, and carbohydrate transport in both mammalian and bacterial cells has been reviewed recently (1, 2, 3, 4, 5); and 2) the influence of air contaminants on membrane function.

A. Terminology

1. Active transport is defined as the net movement of a solute against an electrochemical gradient with expenditure of metabolic energy. From an operational point of view, an active transport system carries out a three step process: a) binding of a solute to a receptor site; b) translocation of the solute-receptor complex across the plasma membrane; and c) coupling of the process to metabolic energy.

2. Facilitated diffusion is a carrier mediated process of solute uptake which does not require utilization of metabolic energy under normal physiological conditions.

3. Transport system, carrier, membrane carrier are used interchangeably to identify a system, obeying saturation kinetics, which facilitates movement of specific solutes across cytoplasmic membranes. Also signifies the part of the transport system which determines its specificity.

4. Distribution Ratio is defined as the ratio of the concentration of solute in the intracellular water to that in the extracellular water, if and only if the solute is free in the aqueous phase and not metabolizeable by the cell.

5. Steady state signifies the maximum intracellular concentration of the solute which can be attained by the cell under defined conditions of pH, solute concentration, and temperature.

B. Amino Acid Transport in Animal Cells

The first observations pointing to a "concentrative uptake" by animal cells were probably made by Van Slyke and Meyer, who

in 1913 found that amino acids injected intravenously rapidly disappeared from blood (6). The important pioneering work in studying accumulation of amino acids by animal cells was carried out by Christensen and colleagues (7, 8, 9, 10, 11) who tested several types of cells for their ability to concentrate each amino acid out of the whole spectrum of natural amino acids and some of their close analogs. Since Ehrlich ascites carcinoma cells (Ehrlich cells) concentrate all the natural amino acids, and maintain higher distribution ratios than most other cells (12), they have been more extensively employed for studies on amino acid transport than other cells. Although detailed studies have been made of amino acid transport in intestine, kidney, and erythrocytes, the general conception of membrane transport of amino acids is derived from its characterization in the Ehrlich cell (13). Data from other types of cells support the concept that most, if not all, living cells are able to transport at least some amino acids, although different cellular species may differ greatly with respect to the development of particular amino acid transporting systems (2, 4, 5).

B.1. Kinetics of Amino Acid Transport

Transport of most natural amino acids, and analogs of amino acids, appears to be mediated by a membrane carrier that binds the substrate before translocating it from the outer to the inner surface of the plasma membrane. This model is inferred from criteria, such as saturation kinetics, specificity, and competitive inhibition by other amino acids, that are considered to be characteristic of carrier mediated transport (14).

The transport of natural amino acids shows saturation kinetics that often fit the Michaelis Menten relationship, and double reciprocal plots of initial velocity against substrate concentration are linear, at least in the lower ranges of concentration. Since the kinetics of carrier mediated transport are similar to enzyme kinetics, the initial reactions of transport are often represented by the simplified model (15, 16, 17):

$$C + Se \underset{k_3}{\overset{k_1}{\rightleftharpoons}} CS \underset{k_4}{\overset{k_2}{\rightleftharpoons}} C + Si$$

where C represents the carrier, and Se, and Si represent the extra- and intra- cellular concentration of substrate, respectively. CS represents the carrier substrate complex and k (k_1, k_2, k_3, k_4) represents the rate constants.

If the total number of carriers is represented by Ct, the maximal rate of unidirectional influx can be expressed by equation 1:

$$Vmax = k_2 (CtS) \qquad (1)$$

Under defined conditions, Vmax is directly proportional to the number of membrane carriers. The initial rate of transport (Vo),

at a single concentration of substrate, is detemined by equation 2:

$$Vo = Vmax \left(\frac{(S)}{(S) + Km} \right) \qquad (2)$$

where Km is the substrate concentration which gives half maximal velocity. Thus, the initial rate of transport at any single concentration of substrate is determined by the following relationship:

$$Vo = k_2 (CtS) \left(\frac{(S)}{(S) + Km} \right) \qquad (3)$$

From equation 3 it is apparent that the initial rate of influx transport is influenced by three factors: k_2, (CtS), and Km. It is assumed in this discussion that k_2 can be influenced only by exchange diffusion (stimulation of solute flux by the intracellular concentration of solute). The number of membrane carriers are reflected by the initial velocity under different experimental conditions only when k_2 and Km remain constant.

The kinetic equations described above apply to the carrier mediated flux of substrates from the extracellular to the intracellular water. These equations do not include the contribution of simple passive diffusion which occurs when Se > Si.

Christensen and Handlogten (18) have reached the conclusion that amino acids escape from the Ehrlich cell via a carrier mediated route similar to that used for uptake. In addition as the concentration of Si increases back diffusion will increase. Thus a set of equations similar to those derived above can be written for the unidirectional flux of substrate from the intracellular water to the extracellular water. Relatively little information is available on the characteristics of carrier mediated efflux. Christensen and Handlogten (18) have demonstrated that the Km values for carrier mediated efflux were, in general, much larger than those for influx, whereas, the Vmax values for the two directions were similar.

These results imply that the "apparent affinity" of the carrier for efflux is much lower than for influx and that under the normal conditions of transport studies (ie., short incubation times) the amount of substrate pumped from the cells may be small.

Therefore the net flux of substrate across the cell membrane is described by:

$$Ms^{e \longrightarrow i}_{net} = Ms^{e \longrightarrow i} - Ms^{i \longrightarrow e}$$

where $Ms^{e \longrightarrow i}$, and $Ms^{i \longrightarrow e}$ represent the movement of substrate from extracellular (e) to intracellular (i), or vice versa.

Each of these unidirectional fluxes is composed of a carrier mediated and passive diffusion component, and the steady state condition will be given by:

$$M^{e \rightarrow i}\ \text{steady state} = \left[k_2 CtS \frac{(Se)}{(Se) + Km} + k_D (Se - Si) \right] - \left[k_4 CtS \frac{Si}{Si + Km} + k_D (Si - Se) \right]$$

where k_D is the diffusion constant of the substrate.

Even in cases of a straight and homogenous Michaelis-Menten relationship the interpretation of Km and Vmax is difficult (14). Km, the half saturation constant, is not an accurate index of the affinity between the carrier and amino acid because Km and Vmax are dependent on the mobility of the loaded and unloaded carrier. If the loaded and unloaded carrier have different rate constants, the initial velocity of transport would be determined by the respective concentrations of loaded and unloaded carrier at any given time. Thus, any factor which influences the ratio of these two rate constants would change Km even though the affinity of the carrier for the substrate is unchanged. In a similar manner, Vmax, which is often interpreted as the product of the total number of carriers, and the rate coefficient for the loaded carrier, may be limited more by the supply of energy (ATP) than by these two factors.

In spite of these reservations about the interpretations of Vo, Vmax, and Km, determinations of these parameters of transport have provided valuable insights into the mechanisms by which hormones (1, 19, 20) and drugs (1) influence transport of amino acids in cells and tissues. These terms will be defined and interpreted in this research project as follows:

1) Initial velocity (Vo): rate of transport at a single concentration of substrate; reflection of the total number of carriers if k_2 and Km are unchanged.

2) Maximum velocity (Vmax): total capacity of the system; reflection of the amount of energy available to the transport system.

3) Michaelis-Menten constant (Km): concentration of substrate which yields half maximal velocity; reflection of "apparent affinity" or "nature" of the carrier.

B.2. Specificities and Special Systems for Transport of Amino Acids

Transport systems, like enzymes, are specific for certain amino acids or groups of amino acids. As a general rule, transport systems are less specific than enzymes, since they only prefer certain amino acids, and there is considerable overlapping specificity between the different systems (1, 21). For example: L-amino acids are transported more rapidly than the D-configuration; neutral amino acids require a free carboxyl and free amino group in the alpha position and a nonpolar side chain; replacement

of the nonpolar group with anionic or cationic groups will abolish the affinity of the amino acid for neutral transport systems, and increase the affinity for a system specific for acidic or basic amino acids.

The most extensive characterization of the specificities of different systems for amino acid transport has been made by Oxender and Christensen (22). Their studies have identified several systems for transport of neutral, basic and acidic amino acids. The characteristics of the "A" system are included in this discussion because alpha amino isobutyric acid (AIB), the amino acid analog used in this research program, is known to be transported by this system.

A system (13)

defined in: Ehrlich Cell
Substrates: all neutral amino acids (alanine, glycine, proline) and analogs of amino acids, amino isobutyric (AIB) and N-methyl amino isobytryric acid (MeAIB)
Vmax: constant
dependency on Na ion: first order; causes one sodium to migrate inward per amino acid molecule
pH sensitivity: barely detectable at pH 5
mechanism: active transport
other apparent sources: Hamster intestine, kidney, brain, hepatoma tissue culture cells; conspicuously absent from mature erythrocytes

Although these kinetic studies have characterized several transport systems in Ehrlich cells for neutral, basic and acidic amino acids the degree to which these systems are represented in normal animal cells remains to be established. Attempts have been made to generalize the transport systems of Ehrlich ascites tumor cells to other mammalian cells. However, several differences in the transport systems for lysine in alveolar macrophages and tumor cells are readily apparent (Section C). The extensive studies of Christensen have provided insights into the transport mechanisms, and offer an invaluable guide for studying transport systems in other types of cells.

B.3. Ions and Amino Acid Transport Systems

One of the most interesting aspects of amino acid transport systems in animal cells is a requirement for extracellular sodium. This sodium dependency is characterized by two distinctive features: 1) if sodium ions are removed from the incubation media the unidirectional influx of the amino acid is decreased, and 2) the steady state concentration of the amino acid in the cell is equal to that in the incubation media. These effects may result from a decrease in maximal flux, an increase in apparent Michaelis-Menten constant (decreased affinity), or both, depending on the type of cell and the amino acid.

The effect of alkali metal ions on the transport of amino acids and sugars has been studied in a variety of cells and tissues (1, 3, 4, 10). In most cells, the data support the hypo-

thesis that sodium ion is a co-substrate for the carrier system: That is, movements of sodium ions down their electrochemical gradient provides the ultimate driving force for active accumulation of amino acids. The alveolar macrophage is an exception, and the anamolous effects of Na+ in this cell are discussed later. There are at least three ways in which sodium ion could act as a co-factor for amino acid transport: 1) facilitation of energy utilization perhaps via Na-dependent adenosine triphosphatase (experimentally measured as an increased Vmax); 2) increasing the affinity of the carrier for the substrate (experimentally measured as a decreased Km); or 3) combination of 1&2.

B.4. Energetics of Transport

Active accumulation of amino acids is an energy dependent process. Inhibitors of oxidative metabolism, such as cyanide, dinitrophenol, or anaerobiosis, inhibit accumulation of amino acids in a variety of cells (1, 23). Usually, these experimental conditions do not completely depress the uptake suggesting, perhaps, that anaerobic glycolysis or other sources of energy are available for active transport. As discussed in Section B.3. the sodium gradient hypothesis, rather than direct coupling of energy to transport, appears to provide the proximate driving force for the intracellular accumulation of amino acids. However, in either case of direct or indirect coupling of metabolic energy to amino acid transport, oxidative metabolism must generate ATP, and it is probable that active transport of amino acids is coupled to both ion gradients and expenditure of metabolic energy. Additional studies concerned with the proportion of ATP utilized for operation of the "Na+ pump" or amino acid transport in a variety of cells are clearly required.

B.5. Chemical Basis for Transport

Most models for transport of amino acids assume that the first step is a specific binding of substrate to a receptor site on a carrier molecule. This binding reaction is then followed by movement of the substrate-carrier complex through the plasma membrane and subsequent release of the substrate inside of the cell. The chemical nature of the carrier molecule remains speculative. In bacterial systems a number of proteins have been proposed as the active carriers involved in transport systems for a variety of amino acids, sugars and ions (5). Unfortunately, similar studies with animal cells has not yet progressed to the same extent.

C.1. Amino Acid Transport in Alveolar Macrophages

Transport of natural amino acids by rabbit alveolar macrophages has been studied extensively by Berlin and colleagues (21). Their studies which used lysine, a dibasic amino acid, and leucine, a neutral amino acid, clearly supported the general conception of a carrier mediated system. However, there are a number of striking differences between the transport systems in alveolar macrophages and Ehrlich cells (24). First, lysine was transported by a single system which had some affinity for all natural amino

acids. In contrast, *Ehrlich cells* have more than one transport system for dibasic amino acids and have no affinity for acidic or neutral amino acids. Second, neutral amino acids, such as leucine or histidine, completely inhibited lysine transport. This observation implied striking differences in specificity between this cell and epithelial tissues where neutral amino acids do not block transport of dibasic amino acids. Third, leucine was transported by at least two systems: one common with lysine, and the other independent of the lysine. Fourth, there was an inverse relationship between sodium and/or potassium and lysine accumulation. Replacement of sodium with potassium, or choline chloride had no inhibitory effect on lysine transport; substitution of sucrose or mannitol for Na^+ caused a 50% stimulation of lysine transport. In Ehrlich cells, reduction in extracellular Na^+ dramatically reduces the accumulation of all amino acids. These anamolous effects of sodium and potassium were associated with a decreased maximal velocity and were not related to changes in apparent affinity of the carrier for the substrate.

As discussed above the influence of sodium ion on active transport of amino acids by animal cells has been largely explained by the effect of Na^+ to increase the affinity of nonelectrolytes to the carrier or to increase the rate of translocation of Na^+ - substrate - carrier complexes across the membrane, or both (3). In addition, the bulk of the evidence supports the concept that the asymmetry of Na ion distribution provides the ultimate driving force for amino acid accumulation. The accumulation of lysine by alveolar macrophages cannot be explained by the sodium gradient hypothesis because the effects of sodium and potassium are equivalent. The physiological significance of these novel cation effects is uncertain and additional studies with other amino acids are required to identify and characterize the systems for transport of amino acids by cells in the lung.

Carrier mediated transport and phagocytosis are two distinctive mechanisms for transporting substances across plasma membranes of macrophages. Data from a recent study of Tsan and Berlin (17) suggested that the membrane sites for phagocytosis and amino acid transport are topographically distinct. In these studies, alveolar macrophages phagocytized polyvinyl toluene (PVT) spheres before measurement of lysine transport. If the transport and phagocytic sites were intermixed a depression in transport should have been observed because large amounts of membrane (more than 50%) are known to be internalized during phagocytosis. However, there was no difference in transport between phagocytically active and resting cells. These results suggested that 1) the two sites were geographically separate, or 2) carrier molecules were rapidly synthesized and inserted into the membrane of the alveolar macrophage.

These possibilities were investigated by treatment of the alveolar macrophages with p-chloromercuribenzene sulfonic acid (PCMBSA). This agent is a powerful sulfhydryl inhibitor (25) that blocks lysine transport (17), but does not kill or penetrate the membrane of macrophages (17). Tsan and Berlin treated alveolar macrophages with PCMBSA before incubation with PVT and measurement of lysine transport. If new carriers were introduced during phagocytosis, the inhibition of lysine transport should have been at

least partially reversed. However, the depression in lysine transport was identical in control and phagocytically active cells indicating that new carriers were not introduced into the membrane of macrophages during phagocytosis.

The impermeability of membranes to PCMBSA makes this compound an ideal tool to quantitate the number of surface sulfhydryl groups (25). Since the inhibition of lysine transport by PCMBSA was prevented by high concentration of substrate it was probable that this compound reacted with -SH groups in the active site of the lysine carrier. Using radiolabeled PCMBSA, the number of membrane -SH groups reacting with the sulfhydryl agent was measured in the presence and absence of high concentrations of lysine. In these experiments, about 13% of the total number of -SH groups in the membrane were responsible for lysine transport. These studies imply that -SH groups in the macrophage membrane are involved in the transport system for lysine. Unfortunately, similar experiments with other amino acids have not yet been performed and it is not possible to state whether active sites of carriers for other amino acids also require sulfhydryl groups. The importance of these studies is that they clearly demonstrate that transport sites, at least for lysine in the alveolar macrophages are topographically distinct from phagocytic sites and that the active site of the lysine carrier contains -SH groups.

Ukena and Berlin (26) have recently examined the role of the microtubule system in maintaining the topographical distribution of lysine carriers in membranes of the polymorphonuclear leukocyte (PMN). Incubation of PMN with colchicine or vinblastine, plant alkaloids which specifically bind to microtubular proteins of mammalian cells and disrupt microtubular function, had little effect on lysine transport. However, when PMN were allowed to phagocytize inert particles in the presence of these drugs, membrane transport of lysine was markedly decreased. These studies suggested that microtubules may be involved in maintaining the topographical distribution of carriers in cellular membranes, or that the alkaloids destroyed the cells.

The relationship between microtubules, active transport and phagocytosis in alveolar macrophages exposed to air contaminants has never been examined. Studies on this relationship would be attractive with cigarette smoke as the contaminant because nicotine is a major component in smoke (27). Although data are not available, it is possible that nicotine, a plant alkaloid, destroys microtubules, and, in a phagocytizing cell, a depression in transport could occur because of the lack of topographical separation of transport and phagocytic sites.

Recent experiments of Berlin have examined the temperature dependence of lysine and adenosine transport systems in alveolar macrophages (28). These experiments revealed a sharp transition temperature in Arrhenius plots of velocity versus the reciprocal of temperature. In bacterial systems, transition temperatures are characteristic of the fatty acids incorporated into the membrane. Sharp transition points are not usually observed in animal cells because cholesterol usually broadens or masks the transition. Although the precise explanation for these transition points is

not certain, they may reflect a transition of long chain fatty acids from a tight, crystalline array to a disordered, more liquid, state. An alternative explanation for transition temperatures in transport systems is that the carrier (protein) exists as an equilibrium between conformers which are favored by high or low temperature, respectively. Conformational changes have also been invoked to account for the transition temperature observed with the Na+, K+ dependent ATP-ase system in membranes (29).

Whether the temperature characteristics of transport are related to phase transitions of lipids or to shifts in the ratio of carrier conformers remains to be seen. However, each mechanism could provide a basis for regulating transport without synthesis or degradation of carrier proteins (28).

II. Alteration of Plasma Membrane Functions by Air Contaminants

Several recent studies have convincingly demonstrated that air contaminants impair the functional properties of alveolar macrophage membranes, as measured by the loss of "adhesiveness," increased uptake of trypan blue, decreased phagocytosis and killing of bacteria, inhibition of membrane bound enzyme activities and impairment of active transport.

A. Permeability Phenomena

Exclusion of vital dyes has been widely used for rapid determination of cellular viability (30, 31). Several investigators have shown that an increased uptake of ionic dyes (trypan blue or eosin y) correlated well with the inability of cells to metabolize substrates or to multiply in tissue culture (32, 33, 34). An increased dye uptake is commonly interpreted as a loss of semipermeability of the membrane and cells that take up dye are referred to as 'dead' cells (30). Air contaminants, such as cigarette smoke (35, 36), aqueous extracts of smoke (37, 38), specific chemicals in smoke (39, 40), ozone (40, 41) or sulfur dioxide (40) have been reported to reduce the ability of cells to exclude trypan blue. In similar studies, I have found the behaviour of alveolar macrophages, incubated in vitro with aqueous extracts of smoke, was equivocal towards trypan blue; some cells contained dye in the nucleus, in the cytoplasm and some in both nucleus and cytoplasm. In addition, in the degree of staining there was variability ranging from light to intense blue. There are a number of objections to dye exclusion as a technique for estimating cellular viability. First, these dyes are normally phagocytized or pinocytized by macrophages (42) and uptake of dye by these cells could be interpreted as a stimulation of membrane activity rather than damage to the membrane. Secondly, this test differentiates damaged from undamaged cells only if the limits of the test are established for the type of cell and the suspending medium being used (30, 43). Since most published reports on dye exclusion porperties of cells do not indicate the dye concentration, serum concentration, incubation time or cell concentration, it is difficult to compare the data. Without prior standardazation, the data obtained with dye

exclusion test are often erratic and difficult to objectively interpret.

Additional qualitative evidence for alterations in the membranes of cells exposed in vitro to environmental contaminants is the observation that cells develop blebs or blister-like formations, visible with the light microscope (39, 41), or detach from the surface of culture vessels (38, 39, 41). Quantitative evidence for loss of membrane integrity is found in the studies of Hurst and Coffin (44) which demonstrated that exposure of macrophages to 2 ppm ozone for 3 hours enhanced the leakage of lysosomal hydrolases and detachment of cells from culture flasks. Addition of glutathione or cysteine to the incubation media eliminated the loss of cell bound enzyme activity, and Hurst and Coffin suggest that -SH groups may be importantly involved in the maintenance of membrane integrity.

These qualitative and quantitative studies suggest that plasma membranes were severely damaged or altered in order for proteases to leak from the cell, or for the cells to detach. If membrane damage is severe enough cells would lyse, and release a broad spectrum of enzymes into the media or onto the surface of the lung. Holt and Keast have recently reported that a single acute exposure of mice to high concentrations of smoke resulted in an immediate and dramatic loss of macrophages in the lung (35). If these data represent lysis of cells, and not an emmigration of cells from the lung, an observation of considerable clinical importance has been made because intratracheal injections of homogenates of leucocytes or alveolar macrophages can induce emphysema-like lesions in the lungs of dogs (45). Therefore a relationship between cigarette smoke induced death of macrophages, with concomitant release of proteolytic enzymes, and development of emphysema in the smokers' lung may be possible. However, the implications of the studies of Holt and Keast are tempered, somewhat, by the studies of Harris et al. which demonstrated a highly significant increase in the number of macrophages in the lungs of human smokers (46).

B. Phagocytosis, Killing and Pinocytosis

Specific functions of alveolar macrophage membranes have also been reported to be influenced by cigarette smoke. Stillman first observed that a heavy dose of cigarette smoke inhibited the clearance of bacteria in mice (49). Since bacterial clearance is primarily a function of the phagocytic activity of alveolar macrophages, Green and colleagues (39, 48), and Lentz and Di Luzio (49) have studied the influence of aqueous extracts of cigarette smoke, or its gas phase, on the in vitro phagocytic activity of rabbit and rat alveolar macrophages. Both groups observed a marked depression in the uptake and killing of bacteria when aqueous extracts or gas phase smoke were added to the incubation media. Interestingly, the toxic effects of smoke could be reduced, in part, by addition of biological antioxidants, such as glutathione or cysteine, to the incubation media. These studies implied that cigarette smoke oxidized reducing substances in the cells and that these reducing substances (i.e. sulfhydryls) were required for phagocytosis, and the activity of intracellular enzymes responsible

for generation of energy for phagocytosis, or production of bactericidal substances.

Schwartz et al. have demonstrated that nicotine, a major component of cigarette smoke, reduced the pinocytotic activity of peritoneal macrophages (50), and enhanced the release of pinolysosomal contents (51). Although the mechanisms for these effects are not known these data suggest that smoke, its chemical components, or other air contaminants severely damage two mechanisms by which alveolar macrophages ingest a variety of substances.

C. Synthetic Activities

Yeager has demonstrated that acute in vitro exposure of M. bovis induced rabbit alveolar macrophages to aqueous extracts of cigarette smoke, or its gas phase, reduced the rate and amount of ^{14}C-leucine incorporated into acid insoluble protein (52). Similar observations have been made by Holt and Keast with short term exposure of cultured alveolar and peritoneal macrophages to whole smoke or its vapor phase (37). However, when cells were maintained in culture for 24 hours, after exposure to smoke, there was no significant difference between the incorporation of ^{3}H-leucine by smoke exposed or control cells. These data, which were interpreted as a "recovery" phenomenon, must be interpreted with caution because the ability of control macrophages, after 24 hours in culture, to incorporate ^{3}H-leucine was decreased by about 70%.

Chronic exposure of macrophages in culture to whole smoke (½, 1, or 2 puffs each day for 4 consecutive days) resulted in a marked dose-dependent response in RNA and protein synthesis (37). At low doses (½, or 1 puff) there was a significant increase in both RNA and protein synthesis; the highest exposure always resulted in a depression of incorporation and viability. The authors claimed that chronic exposure to low doses of smoke lead to the production of metabolically activated cells, and suggested that an increased incorporation signified an adaptative response of cells to a toxic environment. Earlier studies of Holt and Keast demonstrated a marked increase in the incorporation of ^{3}H-uridine into peritoneal and alveolar macrophage RNA thirty minutes after exposure to whole smoke or its gas phase (35). Two patterns of nucleic acid synthesis were observed in macrophages 24 hours after exposure: 1) when more than 20% of the cells were dead, there was a marked increase in incorporation of uridine; and 2) when fewer than 20% of the cells were dead, tritiated RNA synthesis was significantly decreased. These authors suggested that the susceptibility of macrophages (measured by viability and changes in RNA synthesis to cigarette smoke) may be determined, in part, by their basal rate of RNA synthesis.

There are several alternative interpretations to the data of Yeager and Holt and Keast: 1) the decrease in incorporation of labeled amino acids or nucleic acid precursors may reflect a decreased permeability of the macrophage membrane or a decreased activity of membrane transport systems for these substrates; 2) the highly significant increases in protein and nucleic acid synthesis observed after short or long term exposures may result from a transient increase in permeability, carrier activity or cellular metabolism; 3) the depression in incorporation of the labeled

precursors may also reflect alterations in RNA or DNA polymerase activity, stability of messenger RNA for the protein being synthesized, ribosomal stability, or pool sizes of the substrates; 4) uniformly labeled uridine is not an accurate index of RNA synthesis because uridine can be metabolized to cytidine, and incorporated into DNA; 5) "rate" of incorporation implies that the time course of the incorporation was studied when, in fact, Holt and Keast used only a single time of incubation; 6) dead cells, estimated by trypan blue uptake, were not removed from the incubation medium before measurement of incorporation, and one would expect the reported data represented an "average" incorporation by damaged and undamaged cells.

Neither Yeager or Holt and Keast considered these alternative interpretations and until such parameters of substrate incorporation are evaluated the data presented can not be precisely interpreted.

D. Enzyme Activities

The plasma membrane of macrophages plays an important role in the specialized activities of phagocytosis, pinocytosis, chemotaxis and active transport. Migration of macrophages, engulfment of particles, or transport, requires expenditure of energy, and a mechanism for converting the energy of ATP into the mechanical events of translocation. In many cellular systems, an ATP-ase mechanism has been implicated as the membrane bound enzyme system responsible for transduction of chemical energy into work (53). Cross et al. have demonstrated Na^+, K^+, Mg^{++} adenosine triphosphatase activity in plasma membranes of sheep alveolar macrophages (54). Since this enzyme system is membrane bound and an integral part of the ion "pump mechanism", it would appear to be useful as a 'marker' for quantitative studies on the interactions of air contaminants with membranes of cells in the lung. Cigarette smoke is known to contain a number of substances which could inhibit the activity of the membrane ATP-ase system (27). Nicotine (54) and cadmium (56), two components of cigarette smoke, have been shown to inhibit the activity of this enzyme system in alveolar macrophages. Although these studies did not establish a relationship between inhibition of this enzyme system and other functions of membranes, they certainly suggest an attractive hypothesis for evaluating the toxicities of air contaminants.

Recent experiments of York et al. demonstrated an inhibition of cellular respiration by extracts of smoke (59). Since the alveolar macrophage is an obligatory aerobe (58), these studies point out another potential mechanism for the toxic effects of cigarette smoke; that is, inhibition of aerobic metabolism would lead to a deficiency of ATP which, in turn, could produce a depression in phagocytosis, substrate transport, or synthetic activity.

E. Amino Acid Transport in Alveolar Macrophages Exposed to Air Contaminants

Previous studies in this laboratory have demonstrated that aqueous extracts of cigarette smoke (AECS) or nicotine, at concentrations which did not significantly reduce cellular viability, as measured by dye exclusion, markedly depressed the transport of

alpha aminoisobutyric acid (AIB) by rabbit alveolar macrophages (59). Since this material has been submitted for publication, a summary of the significant observations is included here.

Incubation of macrophages with 0.01, 0.05 or 0.1 ml of AECS for 60 minutes resulted in a small but not significant increase in AIB accumulation as measured by intracellular to extracellular ratio greater than controls (Table I). However, higher concentrations of AECS always resulted in a highly significant decrease in accumulation of AIB. The stimulation of membrane transport probably resulted from an increased matabolism; the depression in transport at the higher concentrations is probably related to an alteration in carrier activity of generation of ATP by the cells because there was no significant loss of cells or a marked reduction in cellular viability (Figure I).

AECS (0.5 ml) depressed the rate of accumulation and steady state value of AIB in alveolar macrophages. Since maximal and initial rates of transport, as a function of substrate concentration, were not measured, I could not determine if the capacity of the system, or the nature of the carrier systems were altered by AECS (Figure 2).

Incubation of alveolar macrophages with nicotine, a major component of cigarette smoke, and known to be present in the extract, produced a biphasic response in the accumulation of AIB (Figure 3). Interestingly, the concentrations of nicotine, which produced a significant depression in AIB accumulation, have been previously shown to markedly inhibit cellular respiration and the activity of sodium, potassium, magnesium, ATP-ase in alveolar macrophages prepared from sheep (55, 56). Preliminary studies with cadmium also reveal a similar biphasic effect on the active accumulation of AIB by rabbit alveolar macrophages.

My studies demonstrated that AECS, nicotine or cadmium can depress the accumulation of AIB by alveolar macrophages. The importance of these observations is not yet certain, but I believe these results clearly indicate that air contaminants, at remarkably low concentrations, can adversely affect specific and nonspecific membrane functions of alveolar macrophages, and perhaps other cells in the lung. There are several possible mechanisms by which air contaminants induce alterations in plasma membranes. These include impairment of membrane synthesis, accelerated degradation of membranes, aberrant lipid and or protein synthesis, enzyme inhibition, or peroxidation of membrane lipids. With the exception of lipid peroxidation, there is no information available on the mechanisms by which air contaminants induce membrane alterations. Recently, Delucia et al. (60) have shown that exposure of rats to ozone, an oxidizing air pollutant, decreased the -SH content in membrane, protein, and nonprotein fractions of rat lungs. Although these measurement were not made with macrophages or other cells in the lung, oxidation of cellular reducing substances, such as sulfhydryl compounds, clearly does occur in lungs of experimental animals exposed <u>in vivo</u> to realistic concentrations of air polutants. Recent studies in this laboratory have demonstrated the formation of lipid peroxides in alveolar macrophages after 60 minutes of incubation with aqueous extracts of smoke; addition of cysteine to the incubation media reduced the formation

AMINO ACID TRANSPORT BY ALVEOLAR MACROPHAGES

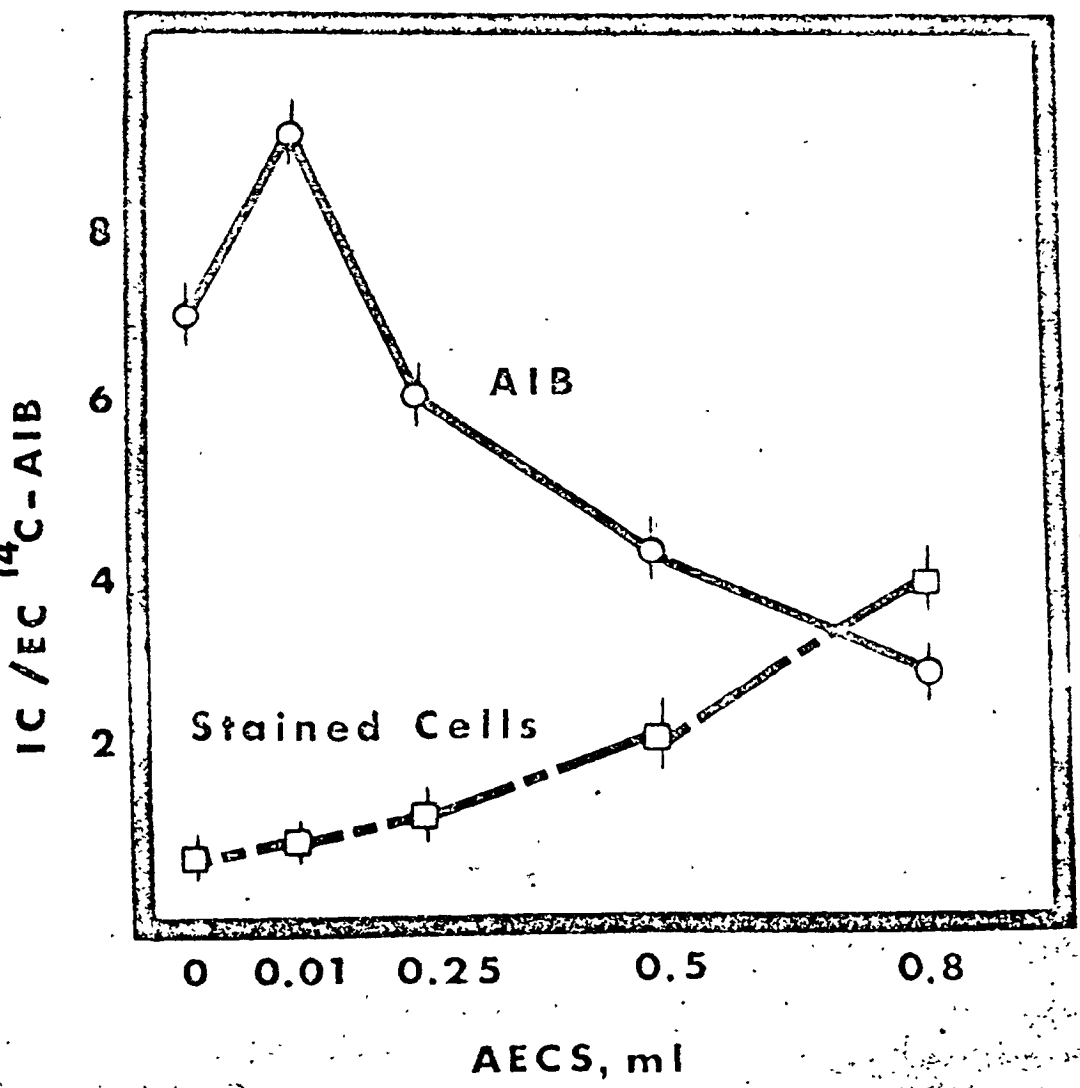
Table I. α-aminoisobutyric acid transport in alveolar macrophages incubated in vitro with aqueous extracts of cigarette smoke.

AECS ml	N	IC/EC (mean ± S.E.M.)	% Change
0	12	6.10 ± 0.54	
0.01	12	6.32 ± 0.97	+7
0.05	10	6.16 ± 1.09	+4
0.10	8	6.93 ± 1.18	+17
0.25	10	4.16 ± 0.65*	-30
0.50	9	2.70 ± 0.53*	-55
0.80	8	1.59 ± 0.38*	-74

Rabbit alveolar macrophages were incubated with ^{14}C-AIB and various concentrations of aqueous extract of cigarette smoke for 1 hour as outlined in materials and methods. N is the number of experiments. * indicates significance of difference from controls calculated by students' "t" test $P \leq .001$.

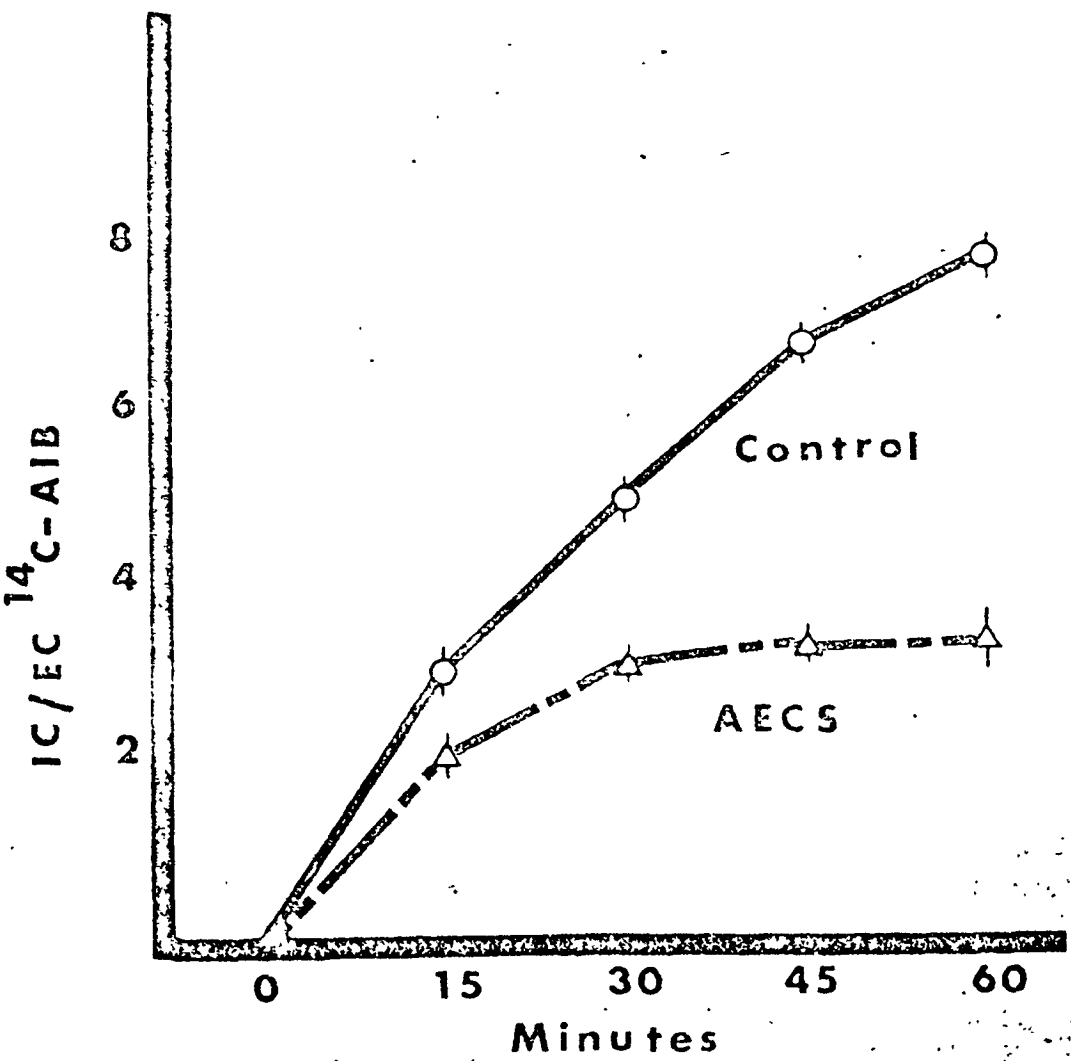
AMINO ACID TRANSPORT BY ALVEOLAR MACROPHAGES

Figure 1. Cells were incubated with ^{14}C-AIB as outlined in Materials and Methods The percent of cells that stained with trypan blue was determined after one hour of incubation with the AECS (broken line). There was no significant change in the total number of cells in the incubation vessel after 60 minutes of contact with the extract. Each point is the mean and range of two experiments with duplicate samples at each concentration.



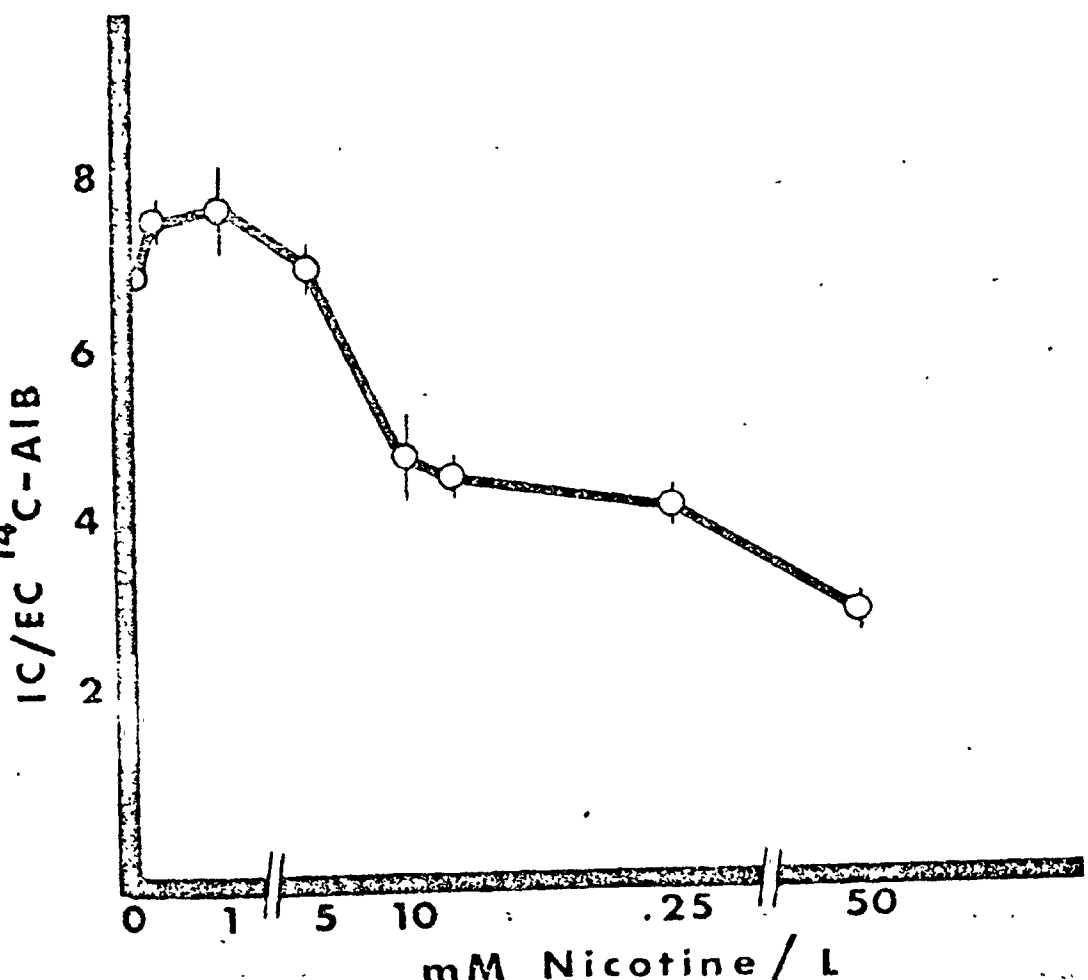
13B

Figure 2. The effect of AECS on the time course of AIB uptake. Rabbit alveolar macrophages were incubated with ^{14}C-AIB for 15, 30 45 and 60 minutes in the presence or absence of 0.5 ml of AECS. Less than 20% of the macrophages incubated with AECS took up trypan blue after 60 minutes of incubation. Each point is the mean and range of 2 experiments with duplicate samples



AMINO ACID TRANSPORT BY ALVEOLAR MACROPHAGES

Figure 3. The effect of nicotine on the transport of AIB by rabbit alveolar macrophages. Alveolar macrophages were incubated with ^{14}C-AIB and various concentrations of nicotine for 1 hour. Each point is the mean and range of 2 experiments with duplicate samples.



of peroxides (61). While these data are far from conclusive, they support the concept that oxidizing air contaminants can reduce the level of reducing agents in cells.

F. Rational For The Proposed Research

Numerous reports in the literature have described the effects of air contaminants on the phagocytic and metabolic activities of macrophages but there are no reports on the influence of cigarette smoke on membrane transport of specific substrates by alveolar macrophages. Preliminary studies in this laboratory have demonstrated that transport of α-amino isobutyric acid (^{14}C-AIB) by alveolar macrophages was adversely affected by concentrations of smoke extract, nicotine or cadmium which had little or no effect on cellular viability as measured by dye exclusion techniques. Since active transport of AIB appears to be a more sensitive and a less equivocal index of plasma membrane integrity than conventional dye exclusion techniques this research program shall investigate the mechanisms by which smoke inhibits active transport in alveolar macrophages.

I have chosen a non-metabolizeable amino acid, α-amino isobutyric acid, as a substrate because it is known to be transported by alveolar macrophages (59), exhibits the transport characteristics of natural amino acids (62), and is not metabolized by the cell (63). Use of such a substrate enables membrane transport to be analyzed in absence of concomittant metabolic changes which are not directly involved in the transport process (67).

III. Methods of Procedure

A. Principle of the Assay System

The theoretical basis for employing active transport as an index of membrane integrity rests on the concept that normal cells will establish and maintain a concentration gradient of the substrate across the membrane (64). This concentration gradient is experimentally measured and expressed as an intracellular to extracellular distribution ratio. When the distribution ratio is greater than unity active transport has occurred; when the ratio is equal to unity no active transport has occurred and simple diffusion can account for the observed distribution ratio. In addition, kinetic analyses can identify the types of transport processes, and suggest whether an agent modifies the number or nature of the membrane carriers.

B. Type of Cells and Incubation System

Rabbit alveolar macrophages will be used because our previous studies (59) have used this macrophage and other investigators (24) have examined the types of transport systems existing in the membrane of the alveolar macrophage. In addition, the large number of cells which can be obtained from a rabbit are more than adequate for a single experiment. An in vitro system for exposing the cells to the test substances will be used because I wish to examine the

direct effects of cigarette smoke on the membranes of alveolar macrophages without the influence of other cell types or invasion of other cells into the lung in response to smoke. Additional advantages of *in vitro* exposures of homogenous cell suspensions over the intact tissue, such as lung slices, are: 1) faster and more complete contact of all cells with the test agents present in the incubation medium (this condition allows decreased reaction times and tends to enlarge the response); 2) a lower complexity of the biological model, represented by a two compartment system (cells, and incubation medium) which simplifies the analysis of the experimental observations, and the mathematical treatment involved.

It will be necessary to examine, in the future, the ability of cigarette smoke to alter amino acid transport after *in vivo* exposures. The primary purpose of these experiments is to examine the mechanism of action of AECS and specific components of AECS on amino acid transport systems in alveolar macorphages in a controlled manner.

C. Metabolic Substrate

14-C-AIB was selected as the substrate because it is known to be transported across the plasma membrane by an energy-dependent, concentrative carrier-mediated mechanism. Following uptake this analogue is neither incorporated into protein nor catatolized; hence its transport can be studied independently of complications introduced by such metabolism.

D. Selection of Test Agents

Three agents have been selected for evaluation on membrane transport: aqueous extracts of cigarette smoke (AECS), nicotine and cadmium. These agents will be used because my previous studies have shown that all three depress the accumulation of ^{14}C-AIB by rabbit alveolar macrophages. In addition, one might expect that water soluble compounds in smoke would dissolve in the aqueous phase covering the surface of macrophages as well as other cells in the lung. Nicotine and cadmium will also be used because both are found in smoke (27), are soluble in aqueous media, easily quantitated, and can depress the activities of sodium, magnesium, potassium activated adenosine triphosphatase localized in the membrane of alveolar macrophages (55, 56). However, nicotine and cadmium are only two of the many agents in smoke (27), and these choices whould not be taken to represent a conclusion that they are the causal agents in the extract.

E. Technical Procedures

1. Preparation of Cells

Alveolar macrophages are obtained by the method of Myrvik et al. (65) as modified by Tsan and Berlin (24) to eliminate contamination with erythrocytes. Alveolar macrophages are sedimented from the lavage fluid by centrifugation at 2000 g-min at 4° . Cell pellets are resuspended in modified Hanks (MH) solution, pH 7.4, containing 5 mM glucose (24). Total number and viability of the alveolar macrophages are determined by incubation of cells (1-2 x 10^6) in 2 ml of MH containing 0.1 ml of 0.5% trypan blue

for 5 minutes at 22° and then counting the total number and the dye containing cells (30, 43). Approximately 1 x 10^8 alveolar macrophages with a viability greater than 95% are routinely obtained from a rabbit with this procedure. More than 90% of the cells so isolated are macrophages as identified by staining characteristics.

2. Preparation of Aqueous Extracts of Cigarette Smoke

Aqueous extracts of cigarette smoke (AECS) are prepared from 100 mm nonfilter, commercially available cigarettes with a Filtrona Smoking Machine (Cigarette Components, Ltd., Middlesex, England). I shall use an aqueous extract of cigarette smoke because previous studies (39, 49) have demonstrated that substances responsible for inhibition of phagocytosis, and killing of bacteria are located in the aqueous phase. Standard settings of a single 25 cc puff of two second duration once each minute are used in all experiments. Aqueous extracts of cigarette smoke are prepared by drawing the smoke from three cigarettes into 25 ml of ice chilled MH. All extracts are filtered through 0.45 u millipore filter and adjusted to pH 7.4 with 0.1 N NaOH immediately before use.

3. Quantitation of Smoke Extracts

A common problem in examining the influence of gas phase smoke, whole smoke or aqueous extracts of smoke on cells is the absence of any quantitation of the smoke in the incubation media. In these studies the dry weight, and the concentrations of nicotine or cadmium will be determined in each extract. Dry weights of the extract will be made on samples of AECS evaporated to dryness at 45°. Alkaloids will be extracted from the extract with chloroform by the procedure of Welcher (66). This procedure, currently used in this laboratory, involves the extraction of alkaloids from pH 9 AECS and evaporation of the chloroform at 45°. Preliminary studies have revealed that 90% of the alkaloids are removed from the extract by two 10 minute extractions, with shaking, at room temperature.

The alkaloid residue is then dissolved in 0.5 N H_2SO_4 and the absorption at 259 mu measured. The concentration of nicotine is then calculated from standard curves relating absorbance to concentration of nicotine.

Cadmium concentration in the AECS will be measured by atomic absorption spectophotometry (67). Dr. Morris Spirtes, Adjunct Professor of Physiology, will perform these determinations.

These data on each extract will permit us to quantitate two of the components present in the extract, and allow us to determine if either agent is totally responsible for the observed depression in accumulation of ^{14}C-AIB by alveolar macrophages caused by AECS.

4. Amino Acid Transport

Alveolar macrophages, suspended in modified Hanks, are incubated at 37° in siliconized 15 x 150 mm glass culture tubes. Each tube contains 5 x 10^6 macrophages, 0.2 uM ^{12}C-AIB, 0.2 µCi

^{14}C-AIB and modified Hanks in a final volume of 2 ml. All incubations are performed at 37° with gentle shaking (10-25 cycles/min) in a Dubnoff shaking incubator with air as gas phase. AECS, nicotine or cadmium in modified Hanks will be added at times and in amounts warranted by each experiment. All tubes are preincubated for 5 minutes before addition of the AIB to ensure temperature equilibration. At termination of the incubation, each cell suspension is transferred to a previously chilled tube and centrifuged at 2000 g- minutes. Each supernatant is transferred to a graduated tube, and the pellets washed twice with 2 ml of MH containing 0.1 uM ^{12}C-AIB/ml. The supernatants from each wash are combined with the first supernatant. Each cell pellet is resuspended in 0.5 ml distilled water (or 0.3 m KOH when protein is determined), transferred to a scintillation vial containing 10 ml Aquasol (New England Nuclear), and counted in a liquid scintillation counter. An aliquot of the thoroughly mixed supernatant is also counted to determine the amount of radioactivity remaining in the media after incubation. The efficiency of counting in each sample is determined by internal standardization using ^{14}C-toluene as standard, and the dpm calculated.

5. Estimation Of Intracellular and Extracellular Water Content

Replicate treated and control cell suspensions are incubated with ^{14}C-inulin in place of ^{14}C-AIB in exactly the same manner as that described for amino acid transport. The wet weight of the pellet is determined and the cell pellet dried at 80° for 18 hours. Total pellet water is calculated by subtracting the dry pellet weight from its wet weight. The dry pellet is then digested in 0.5 ml 0.3 m KOH and the radioactivity of the digested pellet determined. Extracellular water (ECW) is calculated by dividing the total pellet radioactivity by the radioactivity in the incubation medium. Intracellular water (ICW) is then calculated as the difference between total pellet water and extracellular trapped water. Our preliminary studies indicate that intracellular water represents about 70-80% of the total cellular weight. In these experiments we shall always determine if the test agents alter the ICW or ECW during the incubation period.

6. Calculation of Intracellular Amino Acid Content and Expression of Transport Data

The intracellular accumulation of ^{14}C-AIB per ml of intracellular water is calculated from a formula derived by Rosenberg et al. (68):

$$= \frac{Rt - Ao \cdot Ve}{Vt - Ve}$$

where Rt is the net dpm of cell pellet, Ao is the dpm/ml of incubation medium, Ve is volume of extracellular water in ml, Vt is total pellet water in ml.

Results are expressed as either the distribution ratio (Ai/Ao) where Ai is the dpm/ml cell water and Ao radioactivity/ml incubation medium or as umoles AIB per ml of cell water.

7. Measurement of Passive Diffusion, Kinetic Parameters of Transport and Efflux of AIB

The steady state distribution of AIB is determined by the rate of influx and efflux. As previously discussed net influx and net efflux are determined by two factors: passive diffusion and carrier mediated transport. The contribution of non-active transport (diffusion) to the accumulation of AIB is estimated by the method of Akedo and Christensen (62) which involves incubation of macrophages with high concentrations of substrate (40mM), at which the saturable component of AIB transport is presumably saturated. Correction for the diffusion component will be made in all situations in which the distribution ratio approaches 1.

The kinetic parameters of AIB transport are determined by measurement of initial velocity of uptake at different substrate concentrations. Suspensions of cells are incubated for 20 minutes with 0.1 uCi ^{14}C-AIB per ml and unlabelled AIB to give the following final concentrations: 0.1, 0.5, 1.0, 2.5, 5.0, 10, 25, 40 mM. Duplicate samples will be analyzed to obtain distributions ratios. The initial velocity (Vo) is given by the distribution ratio multiplied by the extracellular AIB concentration divided by the incubation time in minutes. The data are plotted as reciprocals of Vo on the y axis, and reciprocals of substrate concentration on the y axis. In this presentation $\frac{1}{Vmax}$ is given by the intercept on the ordinate, and -1/Km by the intercept on the abscissa. Graphic presentation of initial velocity according to the method of Lineweaver and Burk, at AIB concentrations ranging from 0.1 to 40 mM will allow us to estimate the apparent affinity constant, Km, and maximal velocity. Examination of these plots for linearity, will reveal if the transport of AIB is mediated by a single or multiple system. To confirm the kinetic constants obtained with the Lineweaver and Burk representation, these results will also be plotted as Vo vs. Vo/S because this method has been found to give more reliable estimates of apparent Km and Vmax (69).

The rate of efflux of ^{14}C-AIB from alveolar macrophages which have been preloaded with ^{14}C-AIB is measured by determining the rate of loss of tracer from the cells into tracer-free external medium. The volume of the medium is relatively large so that the concentration of ^{14}C-AIB in the medium will be low throughout the experiment. Because of this recycling of the tracer into the cells should be negligible, and the rate of loss of tracer from the cells will be nearly equivalent to the rate of efflux.

Cell suspensions are preloaded with ^{14}C-AIB by incubation at 37° with 0.2 uCi ^{14}C-AIB and 0.2 uM ^{12}C-AIB. After 1 hour incubation the cell suspensions are washed three times with fresh medium and resuspended in 2 ml of AIB-free medium. After 5, 10, 15, 20 and 30 minutes of incubation at 37° duplicate samples are removed, centrifuged immediately to sediment the cells, and the supernatants assayed for radioactivity. The percent of radioactivity appearing in the medium is plotted against time of incubation to obtain an estimate of the amount of radioactivity which leaves the cell.

8. Estimation of ATP Levels in Alveolar Macrophages (19)

A 0.5-ml aliquot of each macrophage suspension is added to 4.5 ml of distilled water and placed in boiling water for 90 s, immediately chilled on melting ice and frozen in plastic tubes at -20° C for up to one week before assay. Macrophage ATP is measured by a modification of the firefly bioluminescence method. Disodium ATP (Sigma Chemical Co.) over a concentration range of 0.1 to 1 uM is used as standard. 0.1 ml of each thawed macrophage lysate, 0.85 ml of Tris-histidine buffer, pH 7.3, containing 80 mM NaCl, 15 mM KCl, 5 mM $MgCl_2$, and 0.05 ml of luciferase previously prepared in distilled water are combined in scintillation counting vials and light emission measured exactly 30 s later in a Nuclear Chicago scintillation spectrometer. All measurements are made in triplicate or quadruplicate.

9. Determination of Sulfhydryl Levels (-SH)

SH levels in alveolar macrophages will be determined by the method developed by Sedlak and Lindsay (70), which determines the total -SH (TSH) in protein and non-protein compounds. The level of protein sulfhydryls is calculated as the difference between total -SH and non-protein sulfhydryls.

IV. Experimental Design

A. Time Course of Accumulation: Effects of AECS, Nicotine or Cadmium

These initial studies are designed to characterize the transport system by which alveolar macrophages accumulate AIB. In all experiments the viability of the macrophage suspensions (estimated by dye exclusion) and stability of cells (estimated by total cell number) will be measured to exclude an excessive number of dead cells or lysis as an explanation for the data.

The time course of AIB accumulation by alveolar macrophages in vitro will be determined by incubation of cell suspensions with 0.2 uM AIB including 0.2 uCi (^{14}C) AIB. Duplicate samples will be analyzed as described previously to obtain distribution ratios after 5, 10, 20, 30, 45, 60, 120, 180, and 240 minutes of incubation. Analysis of data, plotted as distribution ratio vs. time will identify the linear portion of uptake and the apparent steady state distribution ratio. Since alveolar macrophages also have systems which are neither concentrative nor energy requiring, I shall examine the non-active accumulation of ^{14}C-AIB. This passive diffusion component will be determined as described previously.

In order to determine if alveolar macrophages metabolize ^{14}C-AIB under the conditions of incubation used here, cell suspensions willbe incubated with 0.2 uM AIB including 0.2 uCi ^{14}C-AIB for 60 minutes and the amount of $^{14}CO_2$ produced or the amount of ^{14}C amino acid recovered in acid precipitable protein determined. Additional studies will employ silica gel thin layer chromatography to determine if label is recoverable

in species other than ^{14}C-AIB, and to rule out the presense of ^{14}C-AIB in acid soluble proteins.

Since sodium ion is known to markedly influence AIB accumulation in a variety of cells, I shall determine the influence of extracellular sodium ion on the uptake of AIB. For these studies, cell suspensions will be incubated with ^{14}C-AIB for 1 hour in modified Hanks solution containing 36, 72, 144 mM NaCl. Choline chloride will replace NaCl in those solutions which contain less than normal Na+ ions.

Next, the effect of extracellular pH on AIB accumulation will be measured. Cell suspensions will be incubated in modified Hanks solution, pH 6, 6.5, 7.0, 7.4, 8, for 1 hour and the distribution ratios determined.

The energy dependency of AIB accumulation will be demonstrated by addition of sodium cyanide (1×10^{-4}M), an inhibitor of oxidative phosphorylation, to the incubation media. This inhibitor should reduce but not completely abolish the accumulation of AIB by alveolar macrophages.

The kinetic constants of AIB transport by alveolar macrophages will be determined from measurements of initial velocity of uptake at different substrate concentrations as described in Section III E-7.

These control experiments will characterize the AIB transport system in alveolar macrophages and provide the baseline values for examining the effects of the test agents on membrane transport.

Our initial studies are designed to determine the dose response relationship of test agents on accumulation of ^{14}C-AIB by alveolar macrophages. The following parameters of transport: distribution ratio, apparent diffusion constant, K_D, and efflux of AIB, will be determined as a function of incubation time. The concentrations of test agents to be evaluated are:

AECS: 0, 0.01, 0.05, 0.10, 0.25, 0.5, and 0.8 ml/2ml

Nicotine: 0, 0.1, 1, 5, 10, 12.5, 25, 50 mM

$CdCl_2$: 0.05, 0.1, 0.15, 0.2, 0.25, and 0.5 mM

Our preliminary studies have already demonstrated that .25 ml AECS, 25 mM nicotine and 0.15 mM $CdCl_2$ significantly interfere with AIB accumulation. However, these studies did not measure the influence of these agents on efflux of AIB or K_D and these alternate interpretations need to be examined.

From these data it will be possible to construct dose response curves, and these data will identify the concentrations of test agents which interfere with the accumulation of AIB or accelerate the exodus of AIB from the cells. It should also be possible to establish an effective dose (ED 50/60 concentration) for each agent. Dose response data will be analyzed (75) using a logarithmic

transformation for concentration of test agents and a probit transformation for distribution ratios obtained after 1 hour of incubation. A straight line fitted to the resultant points will permit estimation of the concentrations of test agents which reduce the accumulation of AIB by 50% in 60 minutes of incubation (ED 50/60). These data will facilitate comparison of the effective concentrations of each agent required for depression of AIB transport.

Next the influence of test agents on the time course of AIB accumulation will be determined. For these studies cell suspensions are incubated with the ED 50/60 concentrations of AECS, nicotine and cadmium for 5 to 240 minutes. Duplicate samples are removed after various periods of incubation and the distribution ratios determined. From the graphic representation of these data the apparent steady and the linear portion of AIB uptake can be estimated.

B. Effects of AECS, Nicotine, and Cadmium on Kinetic Parameters of AIB Transport

It is generally held that free amino acids are actively transported from the outer to the inner surface of the plasma membrane by a carrier-mediated process which derives energy from ATP. Our previous studies have shown that AECS, nicotine and cadmium impair active accumulation of AIB and these agents could, therefore, inhibit active AIB uptake by limiting the amount of ATP available to support transport or by modifying the nature of the carrier system. Accordingly, these agents might be expected to decrease the total capacity of the system, as estimated by maximal velocity measurements, or by decreasing the apparent affinity of the carrier for the substrate, as estimated by the calculation of Km. Therefore, we shall examine the effects of the test agents on the following kinetic parameters of active transport: initial velocity, maximal velocity, and apparent affinity constant.

For these experiments cell suspensions are incubated with and without ED 50/60 concentrations of AECS, nicotine or cadmium for 15, 30, 60, or 120 minutes before measurement of initial velocities. After each interval of preincubation, cell suspensions are transferred to tubes containing 0.1 uCi ^{14}C-AIB/ml and unlabeled AIB to give a final concentrations of 0.1, 1.0, 5, and 10 mM. After 20 minutes of incubation, duplicate samples are analyzed, as described previously, to determine the distribution ratios. Graphic representation of initial velocity versus substrate concentration according to the method of Lineweaver and Burk will allow us to estimate the apparent affinity constant, Km, and the maximal velocity, Vmax. Examination of the plots for linearity will reveal if the transport of AIB is mediated by a single or multiple system and the type of inhibition observed. If treatment with the test agents depresses Vmax, but fails to change the apparent affinity constant, Km, these data will suggest a decrease in the total capacity of the system.

C. Relationship Between the Effect of AECS, Nicotine or Cadmium on AIB Transport and ATP Content of Alveolar Macrophages

If the experiments described in section 2 indicate that total capacity of the transport system is decreased by incubation of alveolar macrophages with ED 50/60 concentrations of test agents, I shall then examine the following hypotheses:

1. the test agents depress the active accumulation of AIB by reducing the supply of ATP for transport

2. a depression in ATP production will occur simultaneously with or before the depression in AIB accumulation

Cell suspensions are incubated with and without ED 50/60 concentrations of test agents for 5, 15, 30, 60, and 120 minutes before measurement of distribution ratios and cellular ATP levels. Duplicate control and exposed cell flasks are required at each time interval. These studies will indicate the temporal relationship between the level of cellular ATP and AIB accumulation; in addition, these studies will indicate if alterations in AIB transport precede or occur simultaneously with the reduction in ATP concentration.

ATP is required also for continual protein synthesis and these test agents may depress active amino acid accumulation indirectly by blocking synthesis of carrier proteins. Accordingly, we shall examine AIB transport and ATP levels in macrophage suspensions treated with test agents and cycloheximide alone and with both agents simultaneously. Previous studies of Baran (19) have shown that blockage of protein synthesis in thymic lymphocytes with cycloheximide inhibits amino acid transport, a finding which suggests that inhibitors of protein synthesis prevent replacement of labile proteins required for transport. If inhibitors of protein synthesis do not similarly decrease cellular ATP, these data will indicate that this protein is not required for ATP generation. In addition, this observation, which indicates that the effects of the test agents require continuing protein synthesis will be consistent with two possible modes of action: 1) the test agents selectively inhibit the formation of a labile protein involved in transport, or 2) the test agents promote de novo synthesis of inhibitors of transport or a fraudulent protein.

D. Relationship Between the Effect of AECS, Nicotine or Cadmium on AIB Transport and Modification of the Carrier

If the kinetic studies carried out in B suggest that the apparent affinity constant, Km, of the transport system is altered by incubation of alveolar macrophages with the test agents, we shall examine the effects of these agents on the total sulfhydryl levels in alveolar macrophages and attempt to correlate this factor with alterations in AIB transport.

Since Tsan and Berlin (17) have demonstrated that the carrier system for lysine is especially sensitive to a sulfhydryl inhibitor which binds exclusively to membrane -SH groups, we shall attempt

to demonstrate inhibition of AIB transport by PCMBSA. Cell suspensions are incubated with various concentrations of inhibitor (0.001 to 1 mM) for 20 min, rinsed twice, and then the rate of AIB transport determined. If these experiments reveal PCMBSA inhibition of AIB transport, it is probable that SH are required in the active site of the AIB carrier.

Since -SH may be an integral part of the carrier system, we shall determine the levels of nonprotein and protein sulfhydryls in alveolar macrophages incubated with and without ED 50/60 concentrations of the test agents. Cell suspensions will be incubated with test agents for 15, 30, 60 or 120 minutes before determination of distribution ratios and SH contents.

These studies should indicate if there is a temporal relationship between levels of sulfhydryls and AIB transport. If such a relationship is suggested, an important mechanism of action of these three test agents will be apparent.

E. Reversibility of the Effect of AECS, Nicotine or Cadmium on Membrane Transport of AIB

These studies will examine the persistence of the depression in AIB transport induced by the test agents. Macrophages will be incubated with and without ED 50/60 concentrations of test agents for a period of time known to reduce AIB transport. Then the cells will be washed and re-incubated in the absence of the test agent for various intervals of time (15-240 minutes) before measurement of AIB transport, cellular levels of ATP and total sulfhydryl content. These studies will indicate whether the macrophages can recover their ability to transport AIB and the time required for repair.